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Biochemical and Biophysical Research Communications 324 (2004) 937–945
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Diallyl disulfide, a chemopreventive agent in garlic, induces multidrug resistance-associated protein 2 expression

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Received 20 September 2004

Abstract

The organosulfur compounds (OSCs), present in garlic, are studied for their protective effect against human cancers. P-glycoprotein (P-gp) and multidrug resistance protein 2 (Mrp2) are two transporters involved in the defense of cells and in the development of multidrug resistance. Whereas OSCs increase glutathione *S*-transferase activity (GST), Mrp2 plays a role in the transport of glutathione (GSH)-conjugates. In this study, we have investigated the effect of two OSCs, diallyl disulfide (DADS) and *S*-allyl cysteine (SAC), on P-gp and Mrp2 expression in renal brush-border membranes. By Western blot analysis, our results show that DADS induces Mrp2 expression (by 7-fold), which correlates with the rise of GST activity and GSH levels. Surprisingly, a co-administration of OSC with cisplatin, an anticancer drug, significantly increased Mrp2 gene and protein expression (by 30-fold), suggesting that DADS could potentiate the effects of cisplatin. Interestingly, SAC and cisplatin in co-treatment decreased P-gp protein expression and *mdr1b* isoform mRNA levels. In addition, modulation of the *mdr1b* isoform and *Mrp2* by cisplatin was completely abolished by a glutathione precursor, *N*-acetyl cysteine. These results indicate that OSCs present in a garlic-rich diet might alter chemotherapeutic treatments using P-gp or Mrp2 substrates.

Keywords: P-glycoprotein; Canalicular multispecific organic anion transporter; Mrp2; Diallyl disulfide; *S*-Allyl cysteine; Cisplatin

Some natural compounds found in our diet have been shown to possess therapeutic and pharmacologic properties. Among them, *S*-allyl cysteine (SAC) and diallyl disulfide (DADS) are two organosulfur compounds (OSCs) present in garlic (*Allium sativum*) with antitumorigenesis, antiartherosclerosis, and detoxification properties [1–3]. DADS is known for its chemopreventive effects against the development of stomach and colon cancers [4,5]. The activation of detoxification pathways by the induction of phase II enzymes such as glutathione *S*-transferase (GST) is one of the mechanisms proposed for the anticarcinogenic effects of OSCs [6,7]. GST is a detoxification enzyme which catalyzes the conjugation of many electrophile agents and carcinogens with glutathione (GSH) [8]. The GSH-conjugates are often exported from cells by energy-dependent GS-xenobiotic (GS-X) pump also known as the multidrug resistance protein (MRP) [9].

Transport proteins, present in most tissues of the body, are responsible for the distribution and elimination of many clinically important agents. In particular, the transport of xenobiotics is often associated with P-glycoprotein (P-gp) and/or canalicular multispecific organic anion transporter (cMOAT or Mrp2), which are involved in establishing the multidrug resistance phenotype in cancer cells [10,11]. P-gp is a 170 kDa ATP-dependent

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.09.141

transporter which confers resistance by excluding a wide variety of antineoplastic agents from cells [10,12]. P-gp is encoded by two genes in humans, *MDR1* and *MDR3*, and by three genes in rodents, *mdr1a*, *mdr1b*, and *mdr2*. Overexpression of *MDR1*, as well as the *mdr1a* and *mdr1b* gene products, has been implicated in the development of tumor drug resistance. This membrane transporter is also expressed in a variety of normal secretory tissues such as kidney, intestine, and liver, and at high levels in the endothelial cells of brain capillaries [13–15].

Mrp2 was identified in the canalicular membrane of hepatocytes as an ATP-dependent transporter for organic anions that contributes to drug resistance by transporting a wide range of glutathione (GSH), glucuronate, and sulfate conjugates out of cells [16–18]. It was also shown to be present in renal brush-border membranes (BBM) and intestine [19,20]. Resistance to cisplatin is not associated with either P-gp or with MRP1, but a striking correlation was obtained in vitro between cisplatin resistance and Mrp2 expression [11,21]. This anticancer agent forms toxic conjugates in the cell and its use is limited by nephrotoxicity, which is the major side effect of cisplatin aside from the development of drug resistance [22].

We have previously shown that cisplatin induces the renal expression of Pgp and Mrp2 [23]. Since Mrp2 transports GSH-conjugates and that OSCs are known to increase GST and GSH levels, the present study was performed to further investigate the effect of DADS and SAC on both transporters alone or in combination with cisplatin in the kidney. Our results indicate that DADS and SAC modulate the expression of both Mrp2 and P-gp in rat renal BBM.

Materials and methods

Chemicals. Cisplatin and NAC was obtained from Sigma-Aldrich Fine Chemicals (Oakville, ON). DADS was from Fluka (Ronkonkoma, NY) and SAC was a gift from Wakunaga of America (Mission Viejo, CA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ont.). Polyvinylidene difluoride (PVDF) membranes and a Milliblot-Graphite electroblotter I were from Millipore (Mississauga, Ont.). MAb C219 directed against P-gp was from ID Labs (London, Ont.). Anti-mouse and anti-rabbit IgG horseradish peroxidase-linked whole antibodies and enhanced chemiluminescence (ECL) reagents were purchased from Amersham–Pharmacia Biotech (Buckinghamshire, UK). All other reagents were from Sigma-Aldrich Fine Chemicals.

Treatments. Male Sprague–Dawley rats weighing 300–350 g received DADS (200 mg/kg/day) in sesame oil and SAC (200 mg/kg/day) in saline p.o. for 3 days. When cisplatin was co-administered, it was injected as a single subcutaneous dose (5 mg/kg) diluted in saline on day 0. NAC (1 g/kg) in saline was injected as a bolus alone or 30 min before cisplatin. These doses and treatments correspond to standard regimen found in the literature. For each treatment, each group of animals comprised of three rats. Control groups received a single injection of saline and/or daily administration of saline or sesame oil. Rats were sacrificed on day 4 and tissue sampling was performed. Vimentin expression was immunodetected to evaluate the nephrotoxicity. All animal experiments were evaluated and approved by the Institutional Committee for Good Animal Practices (UQAM, Montréal, Que., Canada).

Isolation of renal brush-border membranes. Renal BBM were prepared from control and treated rats. Renal cortices from individual animals were pooled and renal cortex BBM were then isolated by $MgCl_2$ precipitation as previously described [13]. Purified membranes were resuspended in 50 mM mannitol, 20 mM Hepes–Tris, pH 7.5, and stored at −80 °C. Protein content was determined in all experiments with the Coomassie Plus protein assay (Pierce, Rockford, IL).

GST activity and non-protein bound thiol concentration assays. Renal cortex homogenates from control and treated rats were centrifuged at 3000*g* for 10 min at 4 °C to eliminate aggregates. GST activity was measured in the supernatants using a spectrophotometric method with 96-well microplates. Supernatants were incubated in phosphate buffer (0.1 mM, pH 6.5) containing reduced GSH (1 mM) and 1-chloro-2, 4-dinitrobenzene (1 mM). The GST activity was measured by monitoring the absorbance at 340 nm. To determine the non-protein bound thiol concentration, renal cortex homogenates from control and treated rats were incubated with ice-cold 5% TCA containing 1 mM EDTA and centrifuged at 10,000*g* for 10 min. Non-protein bound thiol concentrations in the supernatants were determined in phosphate buffer using 0.1 mM of 5,5′-dithio-bis-2-nitrobenzoic acid in phosphate buffer (0.1 M, pH 7.0). The absorbance of the resulting yellow product was measured at 410 nm.

Detection of P-gp, Mrp2, and vimentin. P-gp and Mrp2 were detected by Western blot analysis. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed. Membranes were resuspended in sample buffer to a final concentration of 1 mg/ml and loaded on 6.25% or 7.5% acrylamide–bisacrylamide (29.1:0.9) gels without prior heating. P-gp was detected using mAb C219 as described previously [13] while Mrp2 was evaluated using a pAb directed against its C-terminal portion, as previously described [23]. Vimentin was immunodetected with an antibody directed against full-length vimentin of porcine origin. Horseradish peroxidase-conjugated antibodies directed against mouse and rabbit IgGs were used as secondary antibodies. Detection was performed with ECL reagents according to the manufacturer's instructions.

RT-PCR. Total RNA was isolated from renal cortex with Trizol reagent (Gibco-BRL, Burlington, Ont.) according to the manufacturer's instructions. RNA was subsequently amplified with the Master Amp RT-PCR kit. Single-stranded cDNA was synthesized from 1 μg mRNA using 0.25 μM primers as previously described [24–26] and 2.5 U of RetroAmp RT DNA polymerase in RT-PCR buffer (MasterAmp, Madison, WI) using a total volume of 50 μl. This buffer also contained 3 mM $MgCl_2$, 0.5 mM $MnSO_4$, and 0.4 mM of each dNTP. The primers used are shown in Table 1. Reverse transcription was run for 20 min at 60 °C followed by 2 min at 94 °C. PCR amplifications of mdr1a, mdr1b, mdr2, spgp, mrp2, and GAPDH cDNAs were carried out for a number of cycles (25, 30, and 35) of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, followed by a final step at 72 °C for 5 min. The PCR products were separated by 1% agarose gel electrophoresis and visualized by UV light in the presence of ethidium bromide.

Densitometric and statistical analyses. The intensities of the bands obtained from Western blots were estimated with a laser densitometer from Molecular Dynamics (Sunnyvale, CA). Statistical analyses were made with Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

GST activity and non-protein bound thiol concentration increase with OSCs administration

To characterize the action of OSCs on detoxification enzymes, GST activity and the non-protein bound thiol

Table 1
Sequence of amplification primers for rat genes

Genes	Primers	Expected size of PCR products (bp)
mdr1a	S: 5′-GATGGAATTGATAATGTGGACA-3′ AS: 5′-AAGGATCAGGAACAATAAA-3′	351
mdr1b	S: 5′-GAAATAATGCTTATGAATCCCAAAG-3′ AS: 5′-GGTTTCATGGTCGTCGTCTCTTGA-3′	325
mdr2	S: 5′-AAGAATTTGAAGTTGAGCTAAGTGA-3′ AS: 5′-TGGTTTCCACATCCAGCCTAT-3′	143
spgp	S: 5′-GAGGTTACTTAATAGCCTACG-3′ AS: 5′-CATCTATCATCACAGTTCCC-3′	413
mrp2	S: 5′-GGCTGAGTGCTTGGAC-3′ AS: 5′-CTTCTGACGTCATCCTCAC-3′	789
GAPDH	S: 5′-CCATCACCATCTTCCAGGAG-3′ AS: 5′-CCTGCTTCACCACCTTCTTG-3′	576

concentration (which mainly represents the level of reduced GSH) in renal cortex homogenates were evaluated (Fig. 1). Rats were treated with DADS, SAC or NAC with or without cisplatin and GST activity was measured. DADS significantly increased GST activity (Fig. 1A). The increase of GST activity reached about 48% and 66% in kidneys from rats treated with DADS alone or in combination with cisplatin, respectively. The activity of GST was unaffected by either SAC or *N*-acetyl cysteine (NAC), a precursor of GSH (Fig. 1A). The non-protein bound thiol concentration was also increased in renal cortex homogenates after specific treatments. Cisplatin and DADS, each administered alone, increased non-protein bound thiol levels by 40% and 68%, respectively (Fig. 1B). Increases in non-protein bound thiol levels were also observed in renal cortex from rats co-treated with cisplatin and either DADS or SAC. Non-protein bound thiol levels tended to remain at normal values in rats co-treated with cisplatin and NAC. Recently, vimentin has been identified as gene marker of cisplatin nephrotoxicity [27]. To evaluate the nephrotoxicity induced by cisplatin treatment, vimentin was immunodetected (Fig. 1C). The results indicate that the dose and the treatment duration of cisplatin did not induce a severe nephrotoxicity because the vimentin expression was unaffected by cisplatin treatment in kidney homogenates. Since Mrp2 transports GSH-conjugates and cisplatin modulates its renal expression as well as P-gp expression [23,28], the effects of these OSCs on both transporters were evaluated alone or in combination with cisplatin.

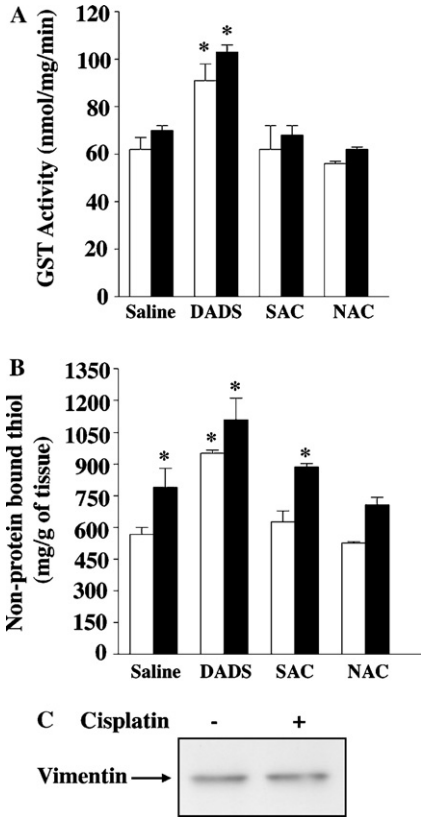

Fig. 1. GST activity and non-protein bound thiol concentrations in kidney increase with OSCs treatment in kidney homogenate. (A) GST activity was measured in various kidney homogenates isolated from control with or without OSCs (open bars) in combination with cisplatin (solid bars). (B) Non-protein bound thiol concentrations were obtained from various kidney homogenates isolated from controls and treated rats with OSCs (open bars) and with cisplatin (solid bars). Each treatment on rats was repeated three times. Means were obtained from duplicate assays on every treatment and the asterisk indicates values that are significantly different from levels found in controls without cisplatin ($p < 0.05$). (C) Immunodetection of vimentin by Western blot was performed using a monoclonal antibody as described in Materials and methods. Vimentin expression was detected in renal homogenates (40 μg) from rats treated with or without cisplatin in saline. Each treatment on rats was repeated three times.

DADS induces Mrp2 expression in renal BBM

Rats were treated with DADS, SAC or NAC alone or in combination with cisplatin and Mrp2 levels were immunodetected by Western blots in renal BBM samples (Fig. 2A). Mrp2 levels were quantified by laser densitometry and expressed as a percentage of those in the corresponding control groups (Fig. 2B). Interestingly,

DADS alone produced a 7-fold increase in Mrp2 expression. In contrast, individual administration of SAC or NAC had no effects on the Mrp2 levels in renal BBM. As previously reported, there was a strong increase (10-fold) in the Mrp2 level induced by a single injection of cisplatin [23]. The most striking results were obtained when cisplatin and DADS were coadministered, where the Mrp2 level became 30-fold higher than in the corresponding control. The increase in Mrp2 levels following cisplatin was unchanged by SAC whereas administration of NAC 30 min before cisplatin caused the Mrp2 level to remain similar to the control values, indicating that NAC neutralized the effect of cisplatin. In addition, controls performed with the various vehicles indicated that they had no impact on Mrp2 expression. In fact, Mrp2 levels were similar when rats received saline (either p.o. or s.c.) or sesame oil (p.o.) and the induction of Mrp2 expression by cisplatin was unchanged when rats were cotreated with the various vehicles (Fig. 2C).

Effect of OSCs with or without cisplatin on P-gp expression

P-gp was immunodetected using mAb C219, and was recognized as a 140–180 kDa protein in renal BBM isolated from control and cisplatin-treated rats (Fig. 3). Western blots were also performed using samples from

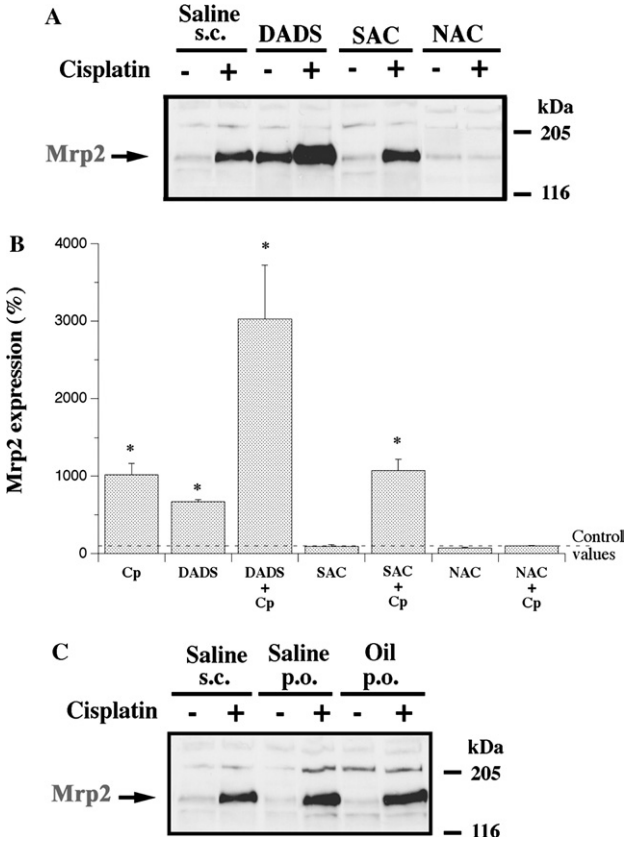

Fig. 2. Immunodetection of Mrp2 in renal BBM after administration of OSCs with or without cisplatin. (A) Immunodetection of Mrp2 by Western blots was performed using a polyclonal antibody as described in Materials and methods. Briefly, Mrp2 was detected in renal BBM proteins (50 μg) isolated from rats treated with cisplatin in saline, with DADS or SAC in sesame oil p.o. or with NAC in saline p.o. and from rats co-treated with each of these products along with cisplatin. (B) Quantification of Mrp2 expression in renal BBM following OSCs in combination with cisplatin. Immunoreactive protein bands detected by Western blot analysis in (A) were evaluated by laser densitometry. Mrp2 levels are expressed as a percentage of the total amount of immunoreactive protein present in control samples. (C) Mrp2 was detected in renal BBM isolated from rats treated with the various vehicles used for OSCs administration (saline s.c.; saline p.o.; and oil p.o.) with (+) or without (−) cisplatin. Each treatment on rats was repeated three times and values represent means ± SE obtained from duplicate assays on every treatment. The asterisk denotes levels significantly higher than the control group ($p < 0.05$).

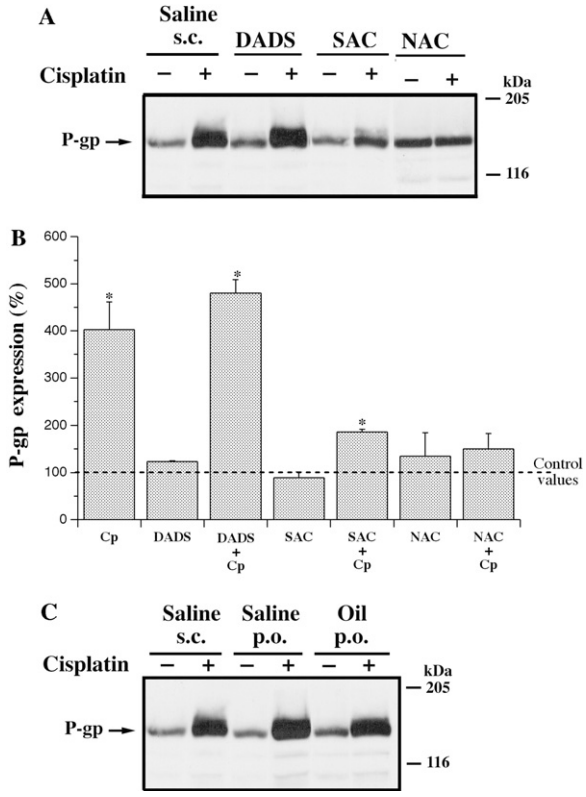

Fig. 3. Effects of OSCs treatments in combination with cisplatin on renal P-gp content. (A) Immunodetection of P-gp was performed using mAb C219 as described in Materials and methods. P-gp was detected in renal BBM (50 μg) isolated from rats treated with cisplatin in saline, DADS or SAC in sesame oil p.o., from rats treated with NAC in saline p.o. and from rats treated with each of these products in combination with cisplatin. (B) Quantification of P-gp expression in renal BBM after administration of OSCs in combination with cisplatin. Immunoreactive protein bands detected by Western blot analysis in (A) were evaluated by laser densitometry. P-gp levels are expressed as a percentage of the total amount of immunoreactive protein present in control samples. (C) P-gp was also detected in renal BBM proteins from rats treated with the vehicles used for OSCs administration (saline s.c.; saline p.o.; oil p.o.) with (+) or without cisplatin (−). Each treatment on rats was repeated three times and values represent means ± SE obtained from duplicate assays on every treatment. The asterisk denotes levels significantly higher than the control group ($p < 0.05$).

rats which had been treated with OSCs with or without cisplatin (Fig. 3A), and their levels of P-gp were expressed as a percentage of the corresponding control groups (Fig. 3B). In contrast to Mrp2 expression, independent DADS, SAC or NAC administration had no effect on renal P-gp expression. A 4-fold increase in the P-gp level was caused by a single injection of cisplatin, which was non significantly potentiated by cotreatment with DADS. However, when SAC or NAC were given to rats in combination with cisplatin, both of these molecules reduced the effects of cisplatin on P-gp expression by 66% and 100%, respectively. Moreover, the vehicles used had no effect on P-gp expression since similar increases in P-gp content were detected following their co-administration with cisplatin (Fig. 3C).

Effect of OSCs and cisplatin on membrane markers

To verify the membrane integrity, the activities of two membrane enzymes were measured in renal BBM isolated from control and treated rats (Table 2). Alkaline phosphatase activities in kidney were unaffected by cisplatin, SAC or NAC alone, or in combination with cisplatin. However, alkaline phosphatase activity increased after DADS administration by 86%, but remained at control values when cisplatin was co-administered. γ-Glutamyltranspeptidase activity was unaffected by the different treatments. These results suggest that the treatments used do not affect the physicochemical properties of BBM which are used for their isolation and that the different levels of expression measured for P-gp and Mrp2 are not related to a general degradation of membrane integrity.

OSCs modulate P-gp mRNA levels in renal cortex

Since mAb C219 recognizes all P-gp isoforms (*mdr1a*, *mdr1b*, and *mdr2*) and probably also the sister of P-glycoprotein (spgp) [29], the expression of individual P-gp proteins was assessed at the RNA level in renal tissue isolated from control and treated rats by RT-PCR analysis with primers specific for the *mdr1a*, *mdr1b*, *mdr2*, and *spgp* cDNAs (Fig. 4). RT-PCR analysis was first

Table 2
Effect of cisplatin and natural compounds on renal membrane markers

Tissue	Alkaline phosphatase (μmol/min/mg)		γ-Glutamyltranspeptidase (μmol/min/mg)	
	− Cisplatin	+ Cisplatin	− Cisplatin	+ Cisplatin
Renal BBM				
Control	4.8 ± 0.9	4.5 ± 0.9	10.0 ± 0.4	8.2 ± 0.4
DADS	8.9 ± 0.3*	6.1 ± 0.5	11.3 ± 0.5	9.3 ± 0.3
SAC	4.9 ± 0.5	4.5 ± 0.7	8.9 ± 0.9	9.3 ± 0.8
NAC	5.9 ± 0.2	6.8 ± 0.2	8.6 ± 0.2	8.4 ± 0.4

Alkaline phosphatase and γ-glutamyltranspeptidase activities were measured at 37 °C using *p*-nitrophenol phosphate and γ-glutamyl-*p*-nitroanilide as substrates with renal BBM isolated from controls and from treated rats. Each treatment on rats was repeated three times and values represent means ± SE obtained from duplicate assays on each treatment. Control values are indicated and the asterisk indicates values that are significantly different from levels found in controls without cisplatin, which are indicated ($p < 0.05$).

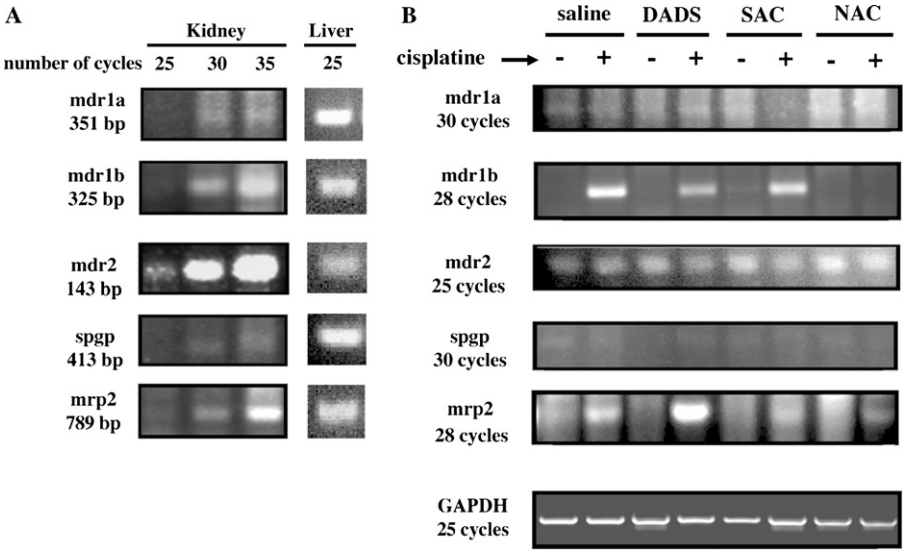

Fig. 4. RT-PCR analysis of renal MDR transporters following OSCs and cisplatin treatments. (A) RT-PCR analysis was performed as described in Materials and methods with specific primers for *mdr1a*, *mdr1b*, *mdr2*, *spgp*, and *mrp2* for varying numbers of cycles. (B) Total renal cortex RNA was also isolated from rats treated with the vehicles used for organosulfur compound administration with (+) or without cisplatin (−). RT-PCR analysis was then performed for these MDR transporters.

performed as a function of PCR cycle number (Fig. 4A). Based on the intensity of observed bands, the *mdr1a*, *mdr1b*, and *mdr2* mRNA were all present in renal cortex whereas the mRNA of *spgp* was almost undetectable. Mrp2 was also detected in the kidney, but at much lower levels than in liver (Fig. 4A). Because cisplatin and OSCs strongly increased the expression of P-gp and Mrp2, RT-PCR analysis of these MDR transporters was performed at the lowest number of cycles that allows evaluation of their mRNA levels (Fig. 4B). Administration of DADS, SAC or NAC alone weakly affected mRNA of these transporters. Cisplatin treatment increased the levels of *mdr1b* and *Mrp2* mRNA whereas *mdr1a*, *mdr2*, and *spgp* mRNAs were unaffected by this drug. The highest increase in *Mrp2* mRNA levels was observed when DADS was co-administered with cisplatin. In addition, the effects of cisplatin on both *mdr1b* and *Mrp2* mRNA were completely abolished when NAC was injected prior to the administration of cisplatin.

Discussion

Membrane transporters play a key role in the defense of cells against anticancer drugs. P-gp and Mrp2 act as energy-dependent drug efflux pumps, reducing the intracellular concentration of unrelated drugs. Cisplatin (5 mg/kg) is largely used for the treatment of solid tumors, however, this drug is associated with high nephrotoxicity. This dose corresponds to chemotherapeutic levels known to induce mild renal failure in rats [30,31]. The stability of vimentin expression 4 days after cisplatin administration indicates weak nephrotoxicity. The expression of this gene can increase up to 2-fold when the cisplatin induces severe nephrotoxicity [27]. Also, the GST activity, the alkaline phosphatase and γ-glutamyltranspeptidase activities remained unaffected by drug administration, suggesting that cisplatin had a mild effect on the kidney BBM 4 days after its administration. In the present study, we further investigated the effect of OSCs, natural agents present in garlic-rich diet, alone or in combination with cisplatin on the endogenous expression of P-gp and Mrp2 in normal tissues. To compare garlic effects consumed daily in our diet, the rats have received OSCs every day for 3 days. Our results show that DADS administration caused the strongest increase in GST activity and in non-protein bound thiols. These data are in agreement with previous reports in which DADS increased GST-α and GST-π in kidney [32,33]. In contrast, SAC and NAC administration did not change GST activity. The difference in GST activity could be explained by chemical structures of compounds tested. Whereas DADS possesses an allyl group with a central disulfide chain, a cysteine is found in addition to allyl group in SAC structure. Recently, a study has demonstrated that the presence of terminal allyl group and central disulfide chain is necessary to induce GST activity by OSCs [6].

Interestingly, Mrp2 expression was increased by DADS administration. Of all the co-treatments administered, DADS and cisplatin had the strongest effect, causing a 30-fold increase in renal Mrp2 protein. The increase in the Mrp2 level by DADS alone or in combination with cisplatin could be to correlate with the increase in non-protein bound thiols. The effects of DADS on renal Mrp2 suggest that the metabolism of the OSCs found in garlic lipids could generate DADS-conjugates with GSH. The existence of such metabolites remains to be established although the metabolism of diallyl sulfide (DAS), a related allyl compound, produced ten DAS–GSH conjugates in bile [34]. Since DAS is a minor constituent (10–20%) of the DADS extract used in the present study, a portion of the effects caused by DADS may be related to DAS–GSH conjugates. However, the identification of the DAS-conjugates suggests that the metabolism of DADS could also generate such GSH-conjugates and that Mrp2 may be involved in the excretion of these metabolites.

In this study, cisplatin alone had no significant effect on GST activity in renal cortex homogenate. Previous studies have associated cisplatin nephrotoxicity with a decrease in GST activity (GST-α, GST-μ levels) in kidney cortex and with an increase in urinary GST activity [35,36]. The stable level of GST activity in renal cortex homogenate in our study may be explained by differences in treatment regimens and doses. Differences in the levels of GST-α, GST-μ, Mrp2 or P-gp and variations in transporter induction due to the use of different rat strains could also explain why GST activity remained unaffected by the drugs used since all these proteins are involved in detoxification processes. In spite of the fact that GST activity was unaffected by cisplatin, the non-protein thiol concentration was increased in renal cortex homogenate. Whereas drop in GSH levels has been measured in severe nephrotoxicity, other studies have measured higher GSH levels following cisplatin treatment [37–39]. The enhancement of glutathione content by cisplatin within mitochondria could prevent damage caused by oxygen free radical by direct detoxification [40]. In tumor cells, the higher levels of GSH have been associated with cisplatin resistance [41]. Moreover, studies showed that cisplatin can form complexes with GSH [42,43]. These complexes could be potentially toxic and might be removed from the cells by a GS-X pump, given the importance to Mrp2-mediated cisplatin resistance [43]. Although the mechanisms of cisplatin active transport have not been fully elucidated, it appears that resistance to cisplatin can be modulated by Mrp2 through transport of the drug, in association with glutathione.

The renal expression of Mrp2 was increased by cisplatin as previously reported [23]. In this previous study, we have showed that the Mrp2 expression induced by cisplatin was maximal between 2 and 6 days after its administration and returned to control values after 15 days [23]. In vitro studies have reported that the GSH is a substrate of Mrp2, which may contribute to cisplatin resistance by exporting the cisplatin–GSH complex [16,44]. In normal rats, about 47% of the initial dose of cisplatin is excreted by the kidney whereas 1–5% is excreted by the liver [45]. The large increase in renal BBM Mrp2 content caused by cisplatin suggests that this transporter may be involved in renal handling or excretion of this drug. Several low- and high-molecular weight cisplatin-bound proteins have been detected [46]. Some of these compounds were isolated from the urine and plasma of the cisplatin-rats by HPLC and one of the metabolites had the same retention time as does an adduct of cisplatin with GSH [47].

The garlic compounds DADS, SAC, and NAC, when administered individually, did not modulate P-gp expression. Only cisplatin treatment induces P-gp expression in renal BBM suggesting that this multidrug transporter may be involved in the renal response to drug cytotoxicity. Also, we have previously demonstrated that the increased P-gp expression by cisplatin in renal BBM was also detected by photolabeling, indicating that P-gp is functional [23]. Modulation of P-gp expression by in vitro exposure to some of its substrate drugs or by chronic administration of the chemosensitizer cyclosporin A (CsA) to rats has also been reported [16,48]. The mechanism involved in the induction of P-gp expression by cisplatin differs from those previously described since it is not a P-gp substrate. As an example of one alternate mechanism, the human Y-Box (YB-1) may be involved in MDR1 gene activation in response to genotoxic stress caused by agents such as UV light and cisplatin [49]. However, SAC reduced the induction of renal P-gp caused by cisplatin whereas NAC completely abolished this induction. It has been recently shown that the allyl compound DAS could be decrease the induced levels of P-gp in resistant cells [50]. The decrease in the induction of P-gp by these two molecules (SAC and NAC) may reflect a diminution of the toxicity associated with cisplatin.

RT-PCR analysis demonstrated that the strongest effect on Mrp2 mRNA level was obtained by DADS and cisplatin cotreatment as observed at protein expression. Mrp2 mRNA levels were unaffected by the administration of DADS alone, suggesting that this organosulfur affects post-transcriptional events. Moreover, RT-PCR analysis showed that ciplatin strongly induced Mrp2 and mdr1b isoform, indicating that this drug affects these MDR transporters at the level of transcription. In contrast, *mdr1a*, *mdr2*, and *spgp* were unaffected by cisplatin. The low level of *spgp* mRNA is in agreement with previous studies showing high levels of *spgp* expression in liver and undetectable levels in kidney [29]. In combination with cisplatin, our data indicated that SAC reduced mRNA level of *mdr1b* isoform. Our results indicate that the effects of cisplatin treatment on renal P-gp and Mrp2 expression are rather specific since two membrane markers, alkaline phosphatase and γ-glutamyltranspeptidase, were unaffected 4 days after cisplatin treatment, as previously reported [44].

Pre-treatment with NAC was reported to reverse cisplatin nephrotoxicity by increasing cisplatin excretion, leading to a lower cisplatin concentration in the kidney [51–53]. It suggested that NAC forms a complex with cisplatin that is not reabsorbed by renal proximal tubules. Our results are in agreement with these studies since administration of NAC prior to cisplatin completely prevented the augmentation of renal Mrp2 and P-gp by cisplatin. The maintenance of both transporter proteins and mRNA levels at control values by prior NAC administration could be related to a reduction of free-cisplatin in renal proximal cells.

In conclusion, these are the first results showing that Mrp2 and the P-gp *mdr1b* isoform are modulated differently in the kidney by OSCs treatment with or without cisplatin. NAC, which is known to reduce cisplatin nephrotoxicity, abolished the effects of cisplatin on both proteins, reinforcing the hypothesis that the increase in Mrp2 and P-gp expression is involved in the response of the kidney to cisplatin. Since cisplatin is a substrate of Mrp2, our results also suggest that Mrp2 could be involved in the secretion of cisplatin–GSH or DADS–GSH conjugates. Together, these results strongly suggest that the chemopreventive agents present in a garlic-rich diet would have a significant effect on chemotherapeutic treatments using P-gp or Mrp2 substrates.

Acknowledgments

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to R.B. J.J. is a recipient of a scholarship from the Canadian Institutes of Health Research.

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